EXHIBIT A

2) Abbreviations used are L-SAM, S-adenosyl-L-methionine; L-SAM-$^{14}CH_3$, S-adenosyl-L-methionine-*methyl*-^{14}C; L-SAH, S-adenosyl-L-homocysteine; STH, S-tubercidinyl-L-homocysteine; 8-aza-SAH, S-8-azaadenosyl-L-homocysteine; N^6-Me-SAH, S-N^6-methyladenosyl-L-homocysteine; N^6-Me_2-SAH, S-N^6-dimethyladenosyl-L-homocysteine; L-SAC, S-adenosyl-L-cysteine; D-SAH, S-adenosyl-D-homocysteine; D-SAH sulfoxide, S-adenosyl-D-homocysteine sulfoxide; N^α-Ac-D-SAH, S-adenosyl-D-N-acetylhomocysteine; COMT, catechol O-methyltransferase (E.C. 2.1.1.6); PNMT, phenylethanolamine N-methyltransferase (E.C. 2.1.1.28); HMT, histamine N-methyltransferase (E.C. 2.1.1.8); HIOMT, hydroxyindole O-methyltransferase (E.C. 2.1.1.4); INMT, indoleethylamine N-methyltransferase; K_{is}, inhibition constant for the slope.

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Potential Inhibitors of S-Adenosylmethionine-Dependent Methyltransferases. 5. Role of the Asymmetric Sulfonium Pole in the Enzymatic Binding of S-Adenosyl-L-methionine

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The configuration at the asymmetric sulfonium pole of S-adenosyl-L-methionine (SAM) necessary for optimal enzymatic binding and methyl donation has been elucidated in this study. For the transmethylations catalyzed by catechol O-methyltransferase, phenylethanolamine N-methyltransferase, histamine N-methyltransferase, and hydroxyindole O-methyltransferase, it was demonstrated that only the natural (−) enantiomer of SAM was active as a methyl donor. The corresponding (+)-SAM, which was prepared by enzymatic resolution of synthetic (±)-SAM, was shown to be inactive as a methyl donor in these enzymatic reactions. The (+)-SAM was found, however, to be a potent inhibitor of each of these enzyme-catalyzed transmethylations. These results suggest that the (+) enantiomer offers a nonproductive configuration for the methyl-transfer reaction itself; however, this configuration fails to hamper enzymatic binding. These results are discussed relative to the geometric requirements necessary for the methyl-transfer reaction and the requirements for enzymatic binding.

For numerous biological transmethylation reactions, the natural methyl donor is S-adenosylmethionine (SAM).[2] Many of the structural features of the amino acid, sugar, and base portions of SAM which are required to produce optimal enzymatic binding and maximal rates of methyl transfer have been elucidated in an accompanying paper in this series.[3] The functional group of fundamental importance in the transmethylation reaction itself is the sulfonium pole. Modifications of the sulfonium center of SAM have primarily involved the replacement of sulfur by selenium[4] and the replacement of the methyl group by an ethyl[4b,5] or by an *n*-propyl[6] group. In addition, earlier studies[7-11] have investigated the role of the configuration of the sulfonium pole in these enzyme-catalyzed transmethylations. Because the absolute configuration of the sulfonium center has not yet been determined, stereoisomers are designated (+) and (−) based on polarimetry. De La Haba et al.[7] have shown that SAM prepared enzymatically from L-methionine and ATP has the (−) configuration and, therefore, is referred to as (−)-L-SAM. SAM chemically synthesized from the methylation of S-adenosyl-L-homocysteine[7] or by condensation of 5'-methylthioadenosine with 2-amino-4-bromobutyric acid[8] is racemic at the sulfonium pole and is referred to as (±)-L-SAM. SAM with the (+) sulfonium configuration [(+)-L-SAM] has been prepared by treatment of (±)-L-SAM with guanidinoacetate methyltransferase (E.C. 2.1.1.2), which selectively utilizes only the (−)-L-SAM as a substrate.[7,10] By using these purified stereoisomers of SAM [(−)-L-SAM, (+)-L-SAM, and (±)-L-SAM], it has been demonstrated that most methyltransferases show a high degree of specificity for the (−) sulfonium configuration in the methyl-transfer reaction itself; e.g., only the (−)-L-SAM was shown to be a substrate for histamine N-methyltransferase (HMT),[11] hydroxyindole O-methyltransferase (HIOMT),[11] catechol O-methyltransferase (COMT),[7] guanidinoacetate methyltransferase,[7,11] and

donor. The one interesting exception to this high specificity pattern is homocysteine *S*-methyltransferase, which is capable of utilizing both the (−)-L-SAM and (+)-L-SAM as substrates.[9,11]

Because our laboratory was investigating analogues of *S*-adenosyl-L-homocysteine (L-SAH)[12-17] and SAM[3] as inhibitors of SAM-dependent methyltransferases, we became interested in determining why (+)-L-SAM was not a substrate for these methyltransferases. Did the (+) configuration at the sulfonium center of SAM adversely affect enzymatic binding or was the geometry offered by this configuration undesirable for methyl transfer to the acceptor substrate? If the geometry offered by the (+) configuration was nonproductive for methyl transfer, but did not adversely affect enzymatic binding, this would offer a simple way to modify SAM, converting it from a biological methyl donor to an inhibitor of methyltransferases. This possibility has been explored using the transmethylations catalyzed by COMT, HMT, HIOMT, and phenylethanolamine *N*-methyltransferase (PNMT) and the results are reported in this paper.

Experimental Section

The general experimental techniques and equipment used in this study were described in a preceding paper in this series.[17] The following compounds are commercially available from the indicated sources: 3,4-dihydroxybenzoate (Aldrich); DL-β-phenylethanolamine, histamine dihydrochloride, *N*-acetylserotonin, (−)-SAM iodide (Sigma); (−)-*S*-adenosyl-L-methionine-*methyl*-^{14}C (SAM-$^{14}CH_3$, 55.0 mCi/mmol), (−)-*S*-adenosyl-L-methionine-*carboxyl*-^{14}C (SAM-$^{14}CO_2H$, 54 mCi/mmol) (New England Nuclear); $^{14}CH_3I$ (55.0 mCi/mmol) (Amersham/Searle). *S*-Adenosyl-L-homocysteine (L-SAH) was synthesized according to a previously described procedure.[18]

(±)-*S*-Adenosyl-L-methionine [(±)-L-SAM]. The (±)-L-SAM was prepared using a modification[3] of the procedure first described by Jamieson.[19] L-SAH (50 mg, 0.13 mmol) was dissolved in formic acid (2 ml) to which was added an excess of methyl iodide (1.0 ml). The reaction mixture was kept stoppered in the dark for 5 days after which ice-cold water (ca. 5 ml) was added and the unreacted methyl iodide extracted with cold Et_2O. The aqueous layer was lyophilized and the residue dissolved in pH 7.0, 0.01 M phosphate buffer. The buffer solution was applied to a column (2 × 8 cm) of Amberlite IRC-50 ion-exchange resin previously equilibrated with 0.01 M phosphate buffer, pH 7.0. After eluting the unreacted SAH with 100 ml of 0.01 M phosphate buffer, pH 7.0, and 50 ml of 0.25 N HOAc, the (±)-SAM was eluted with 50 ml of 4 N acetic acid. The eluate was lyophilized to yield (±)-L-SAM in 80% yield. The (±)-L-SAM was shown to be homogeneous and indistinguishable from commercially available (−)-L-SAM in four thin-layer chromatography systems. Degradation experiments similar to those described earlier by Zappia et al.[20] further confirmed the SAM structure. These experiments included hydrolysis of (±)-L-SAM using 0.1 N NaOH at 100° for 10 min resulting in the formation of adenine and methionine which could be identified by TLC.

(±)-*S*-Adenosyl-L-methionine-*methyl*-^{14}C [(±)-L-SAM-$^{14}CH_3$]. The (±)-L-SAM-$^{14}CH_3$ was prepared using a procedure similar to that described above for the unlabeled (±)-L-SAM. L-SAH (5.0 mg, 0.013 mmol) was dissolved in a mixture of HCOOH (0.5 ml) and glacial HOAc (0.05 ml) which contained 100 μCi of $^{14}CH_3I$ (specific activity 0.5 mCi/mmol). The reaction mixture was allowed to stand in the dark for 5 days after which time the desired (±)-L-SAM-$^{14}CH_3$ was isolated as described above for the unlabeled (±)-L-SAM. The specific activity of the isolated (±)-L-SAM-$^{14}CH_3$ varied slightly from batch to batch but generally was approximately 0.4 mCi/mmol (1000 dpm/nmol). The (±)-L-SAM-$^{14}CH_3$ was characterized by its thin-layer chromatographic properties and degradation experiments.[20]

(+)-*S*-Adenosyl-L-methionine [(+)-L-SAM]. The (±)-L-SAM was enzymatically resolved to yield pure (+)-L-SAM by taking advantage of the substrate specificity of COMT. This enzyme (±)-L-SAM reported here is similar to that described earlier by Jamieson,[19] except for the use of COMT instead of guanidine acetate methyltransferase.

A reaction mixture containing potassium phosphate buffer, pH 7.60, 1500 μmol), 3,4-dihydroxybenzoate (30 μmol), magnesium chloride (18.15 μmol), dithiothreitol (60 μmol), (±)-L-SAM (1 μmol), (−)-L-SAM-$^{14}CO_2H$ (0.25 μCi; specific activity 2.1 mmol), 12 ml of a COMT preparation (specific activity 24.3 of product/mg of protein/min; protein concentration 9.2 and water to a total volume of 24.3 ml was incubated for 1 at 37°. The reaction mixture was then immediately filt through a Millipore filter (HAMK, 25 mm, pore size 0.4 The filtrate was concentrated by lyophilization and the re dissolved in 2 ml of 0.01 M phosphate buffer, pH 7.0 phosphate buffer solution was applied to a column (1 × 16 of Amberlite IRC-50 ion-exchange resin previously equili with 0.01 M phosphate buffer, pH 7.0. The excess 3, hydroxybenzoate, the methylated products, and *S*-adeno L-homocysteine-*carboxyl*-^{14}C (L-SAH-$^{14}CO_2H$) were eluted 100 ml of 0.01 M phosphate buffer, pH 7.0. An interme fraction was eluted with 50 ml of 0.25 N HOAc. The de (+)-L-SAM was then eluted with about 50 ml of 4 N HOAc the resulting eluate was lyophilized. The residue was diss in 1 ml of water and the concentration of (+)-L-SAM determ by the uv absorbance. The yield of pure (+)-L-SAM was gene 2.5–3.5 μmol (45–62%). The resolved (+)-L-SAM was shown be homogenous and chromatographically indistinguishable (±)-L-SAM or (−)-L-SAM in four thin-layer chromatogra systems as well as paper chromatography. Degradation e iments similar to those described earlier by Zappia et al.[20] fu confirmed the SAM structure of the isolated product.

The resolved (+)-L-SAM was not contaminated with significant amount of L-SAH, since no L-SAH was observe TLC or paper chromatography. By including (−)-L-SAM-^{14}C in the incubation mixture, we were able to label the po (−)-L-SAM and also label the pool of SAH (L-SAH-$^{14}CO_2H$) fo during the reaction. This has provided a sensitive meth determine the extent of the reaction [i.e., all of the (−)-L was consumed] and that the isolated (+)-L-SAM was not taminated with L-SAH. In the samples of purified (+)-L only trace amounts of radioactivity were detected indicati at least 98% of the (−)-L-SAM was consumed and that the were free of L-SAH.

Enzyme Isolation and Assay Techniques. The enz used in this study were purified from the following sourc cording to previously described procedures: COMT,[12,21] ra (male, Sprague–Dawley, 180–200 g); PNMT,[12,22] bovine a medulla (Pel-Freez Biologicals); HMT,[12,23] guinea pig (Pel-Freez Biologicals); and HIOMT,[12,24] bovine pineal (Pel-Freez Biologicals). COMT, PNMT, HMT, and HIOM assayed using radiochemical techniques measuring the t of methyl-^{14}C from (−)-L-SAM-$^{14}CH_3$ to the appropriate ac molecules as described in the preceding papers in this seri

For each of the enzyme reactions studied the extent of transfer from (−)-L-SAM-$^{14}CH_3$ or (±)-L-SAM-$^{14}CH_3$ appropriate acceptor molecules was determined. This complished by prolonged incubation of the appropriate the acceptor substrate (250 nmol), and (−)-L-SAM-^{14}CH nmol, 0.05 μCi) [or (±)-L-SAM-$^{14}CH_3$ (12.5 nmol, 0.005 μC monitoring the ^{14}C-labeled product formed by simple ex of the product and counting for radioactivity.[12] In addit ^{14}C-labeled products were separated on paper chromato to confirm the efficiency of the extraction procedures d above and the identity of the products (Figure 2).

Enzyme Kinetics. The (+)-L-SAM prepared in this st tested as an inhibitor of transmethylations catalyzed by PNMT, HMT, and HIOMT from (−)-L-SAM-$^{14}CH_3$ to propriate acceptor molecules. The procedures used to de the inhibition constants (K_i) are identical with those d earlier in our studies of SAH analogue.[12-17] Processin kinetic data was achieved as previously described.[12-17]

Results and Discussion

Preparation of (±)-L-SAM and (+)-L-SAM. (±)-L-SAM and (±)-L-SAM-$^{14}CH_3$ were prepared

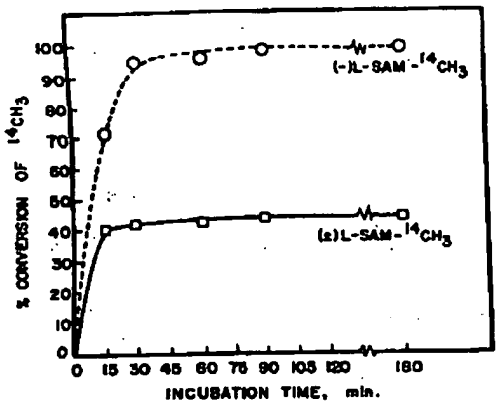


Figure 1. Percent methyl transfer from (−)-L-SAM-$^{14}CH_3$ and (±)-L-SAM-$^{14}CH_3$ to 3,4-dihydroxyacetophenone by COMT. Incubation mixtures were prepared containing 3,4-dihydroxyacetophenone (0.50 μmol), Mg^{++} (0.30 μmol), dithiothreitol (1.0 μmol), COMT preparation (250 μg), phosphate buffer, pH 7.60, (25 μmol), and (−)-L-SAM-$^{14}CH_3$ (0.05 μCi, 0.025 μmol) [or (±)-L-SAM-$^{14}CH_3$ (0.005 μCi, 0.025 μmol)] in a total volume of 0.25 ml. Incubations were carried out for the indicated times at 37° after which the reactions were stopped with 0.10 ml of 1 N HCl. The assay mixtures were extracted with 10 ml of toluene–isoamyl alcohol (7:3), and after centrifugation a 5-ml aliquot of the organic phase was measured for radioactivity. The results were corrected using the appropriate blanks. Percent conversion of methyl-$^{14}CH_3$ was calculated based on the total labeled methyl donor available. Points represent averages of duplicate determinations.

method for preparing (±)-L-SAM was a modification of the procedure first described by Jamieson.[19] These chemically synthesized samples of SAM were racemic at the sulfonium center and the structures were confirmed by comparison with enzymatically prepared (−)-L-SAM with respect to their chromatographic properties, their NMR and uv spectral properties, and by comparison of the products obtained after hydrolysis under basic conditions.[20]

The (+)-L-SAM was prepared by a process of enzymatic resolution, where the strict substrate specificity of COMT was utilized. Shown in Figure 1 is a comparison of the ability of COMT to use (−)-L-SAM or (±)-L-SAM as methyl donors. When this transmethylation reaction was carried out using (−)-L-SAM-$^{14}CH_3$ as a substrate, complete transfer of the methyl-^{14}C from (−)-L-SAM to the product was observed. However, if (±)-L-SAM-$^{14}CH_3$ was used as a substrate, no more than 50% conversion of the methyl-^{14}C to the product was detected. These results are consistent with the earlier observations[7] that methyltransferases, in general, utilize only one of two possible isomers at the sulfonium center. The interpretation of the data for (±)-L-SAM shown in Figure 1 would be that COMT also preferentially utilizes one sulfonium isomer as a methyl donor. This is consistent with data previously reported by De La Haba et al.[7]

To further substantiate these findings, incubation mixtures containing COMT similar to those described in Figure 1 were prepared using either (−)-L-SAM-$^{14}CH_3$ or (±)-L-SAM-$^{14}CH_3$ as substrates and the products characterized by paper chromatography. Prior to incubation, samples were removed and chromatographed on paper to determine the identity of the radioactive material. With both the (−)-L-SAM-$^{14}CH_3$ and (±)-L-SAM-$^{14}CH_3$ incubation mixtures, the radioactivity had the same R_f values as SAM (Figure 2). These reaction mixtures were then incubated at 37° for 180 min after which time another paper chromatogram was run and the results are also

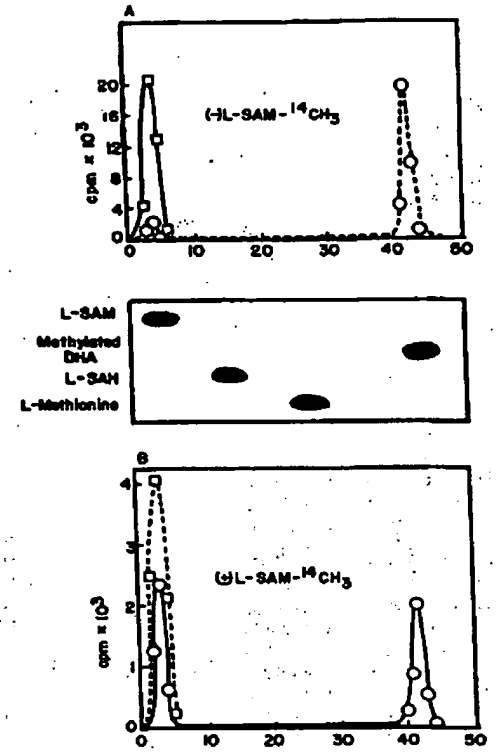


Figure 2. Chromatographic identification of labeled products from the incubation mixtures containing COMT and (−)-L-SAM-$^{14}CH_3$ [or (±)-L-SAM-$^{14}CH_3$]. Incubation mixtures were prepared in a manner identical with that described in Figure 1. Paper chromatography system: 1-butanol–acetic acid–H_2O (12:3:5). The center panel shows the respective chromatographic patterns for L-SAM, L-SAH, methylated DHA, and methionine. (A) Results using (−)-L-SAM-$^{14}CH_3$ as a methyl donor: o–o, aliquot (0.1 ml) removed at 0 time prior to incubation and chromatographed; o–o, aliquot (0.1 ml) removed after incubation at 37° for 180 min and chromatographed. (B) Results using (±)-L-SAM-$^{14}CH_3$ as a methyl donor: o–o, aliquot (0.1 ml) removed at 0 time prior to incubation and chromatographed; o–o, aliquot removed after incubation at 37° for 180 min and then chromatographed.

shown in Figure 2. As can be seen, when (−)-L-SAM-$^{14}CH_3$ was the substrate, all of the radioactivity chromatographed with the methylated products. However, when (±)-L-SAM-$^{14}CH_3$ was the substrate, about 50% of the radioactivity chromatographed with the methylated products and the other 50% with SAM. Further evidence that the radioactivity which chromatographed with SAM was indeed unreacted (+)-L-SAM-$^{14}CH_3$ was obtained by treating an aliquot of this incubation mixture with 0.1 N NaOH at 100° for 10 min and the resulting solution chromatographed on paper (under these conditions sulfonium nucleosides such as L-SAM hydrolyze to methionine and adenine[20]). After hydrolysis of this suspected sample of (+)-L-SAM-$^{14}CH_3$, the radioactivity chromatographed with L-methionine, consistent with the structural assignment.

All of the data described above are compatible with the idea that COMT preferentially utilizes only one isomer of (±)-L-SAM. Taking advantage of this substrate specificity, we have used the COMT-catalyzed reaction to prepare large quantities of the (+)-L-SAM in order to study its inhibitory properties. In these large-scale incubation mixtures, we routinely incorporated a small quantity of (−)-L-SAM-$^{14}CO_2H$, which provided us with a simple way of labeling the pool of (−)-L-SAM. In this way we could

Table I. Inhibition Constants for (+)-L-SAM and L-SAH toward COMT, PNMT, HMT, and HIOMT[a]

Enzyme	Inhibn constants, μM,[b] K_{is} ± SEM	
	(+)-L-SAM	L-SAH[c]
COMT	28.83 ± 3.65	36.3 ± 2.20
PNMT	32.16 ± 13.0	29.0 ± 2.84
HMT	7.35 ± 2.20	18.5 ± 2.19
HIOMT	28.98 ± 5.4	18.5 ± 1.9

[a] COMT, PNMT, HMT, and HIOMT were purified and assayed as described in the Experimental Section. (−)-L-SAM concentrations, 3.3–53.0 μM. [b] Each inhibitor showed linear competitive kinetics and the inhibition constants were calculated as previously described.[11–17] [c] Data taken from ref 12.

determine that (1) the reaction was completed and all of the (−)-L-SAM was consumed, and (2) the isolated (+)-L-SAM was not contaminated with L-SAH. In the purified (+)-L-SAM used in our inhibitory studies, only trace amounts of radioactivity were detectable. These trace levels of radioactivity appear to be attributable to unreacted (−)-L-SAM which amounted to no more than 1–2% of the (+)-L-SAM present in the sample.

Methyl Transfer from (±)-L-SAM Using PNMT, HMT, and HIOMT. We have obtained results for PNMT, HMT, and HIOMT similar to those shown in Figures 1 and 2 for COMT indicating these enzymes also preferentially utilize (−)-L-SAM as a methyl donor. With each of these enzymes no greater than 50% conversion of the labeled methyl group of (±)-L-SAM-$^{14}CH_3$ to the appropriate acceptor molecule could be detected. This was further confirmed by extensively incubating purified samples of (+)-L-SAM-$^{14}CH_3$ with these enzymes in an effort to detect any possible methyl donor properties. However, using (−)-L-SAM-$^{14}CH_3$ as a methyl donor, complete transfer (100%) of the labeled methyl group to the acceptor molecule was observed with each of the enzymes tested. Results similar to these had been reported earlier for HMT and HIOMT.[6]

Inhibitory Activity of (+)-L-SAM. Having available sufficient quantities of (+)-L-SAM, we were interested in determining whether this isomer was inactive as a methyl donor because it failed to bind to the enzymes or because it bound with an orientation of the methyl group that did not permit transfer to the acceptor substrate. Therefore, the inhibitory properties of (+)-L-SAM were studied using the COMT, PNMT, HMT, and HIOMT catalyzed reactions from (−)-L-SAM-$^{14}CH_3$ to the appropriate acceptor nolecules. Preliminary experiments showed that (+)-L-3AM had potent inhibitory effects on these enzymatic transmethylations. Using reciprocal velocity vs. reciprocal (−)-L-SAM plots, the kinetic patterns for inhibition of COMT, PNMT, HMT, and HIOMT by (+)-L-SAM were determined and the resulting inhibition constants are listed in Table I. In all cases linear competitive patterns of inhibition were observed when (−)-L-SAM was the variable substrate. For example, in Figure 3 is shown the kinetic pattern for inhibition of PNMT by (+)-L-SAM. The linear competitive kinetic patterns suggest that the binding sites for (+)-L-SAM are identical with the (−)-L-SAM binding sites. For comparison, the inhibition constants (K_{is}) for L-SAH are also provided in Table I. It is extremely interesting to note that (+)-L-SAM shows inhibitory activities toward these four enzymes comparable to L-SAH. Therefore, from these results it is apparent that the lack of methyl donor compatibility of (+)-L-SAM resides in the misorientation of the methyl group at the sulfonium center

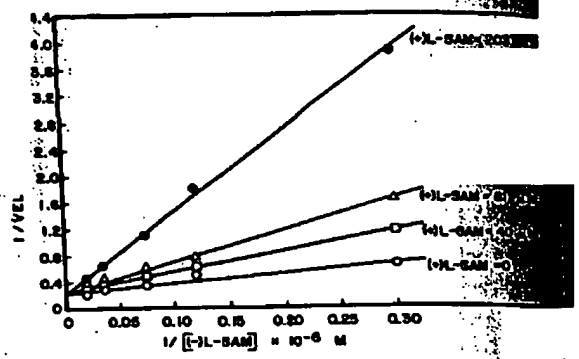
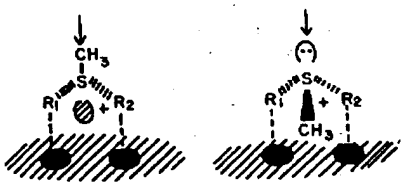


Figure 3. (+)-L-SAM inhibition of PNMT. Reciprocal plots with (−)-L-SAM as the variable substrate. Assay conditions were outlined in the Experimental Section except (−)-L-SAM concentration, 3.3–53.0 μM [(−)-L-SAM-$^{14}CH_3$ = 0.05 μCi]. DL-β-Phenylethanolamine concentration, 1.0 mM. Vel = nmol of product/mg of protein/min. Points represent averages of duplicate determinations.

Chart I. Possible Configurations of the Sulfonium Center of L-SAM[a]



[a] R_1 = -$CH_2CH_2CH(NH_2)CO_2H$ (L); R_2 = 5'-adenosyl. Absolute configurations of sulfonium center are unknown. Arrow denotes predetermined approach of an enzymatically bound nucleophile.

methyl group, however, does not appear to adversely affect enzymatic binding, since (+)-L-SAM is a potent inhibitor of these enzymes.

Conclusion

We have attempted in this study to determine the specificity of COMT, PNMT, HMT, and HIOMT for configuration at the sulfonium center of the methionine moiety of L-SAM. Earlier studies[7–11] have shown that COMT, PNMT, and HIOMT utilize only (−)-L-SAM as a methyl donor and not the corresponding (+)-L-SAM. In this study we have confirmed these findings for COMT, HMT, and HIOMT and, in addition, have shown that PNMT exhibits a preference for the (−) isomer of L-SAM as a substrate. With PNMT, (+)-L-SAM showed no methyl-donating properties.

Of primary concern in this study was to determine if (+)-L-SAM was inactive as substrate because it failed to bind to the enzyme or if it was inactive because the stereometry offered by this configuration was undesirable for methyl transfer to the acceptor substrate. To answer this question we prepared pure (+)-L-SAM by enzymatic resolution of (±)-L-SAM utilizing the substrate specificity of COMT. This pure (+)-L-SAM was found to be a potent inhibitor of COMT, PNMT, HMT, and HIOMT. These observations clearly demonstrate that the enzymes studied have a high affinity for (+)-L-SAM indicating that the configuration of the sulfonium center does not adversely affect enzymatic binding. However, the configuration of the sulfonium center of (+)-L-SAM must be nonproductive for methyl transfer, since this isomer shows no methyl donating abilities. Shown in Chart I are the two [illegible]

amino acid moiety is depicted as R_1 and the adenosyl moiety is depicted by R_2. In earlier studies from our laboratory we have shown that there are functional groups crucial for enzymatic binding on the amino acid, sugar, and base portions of L-SAH[12-17] and L-SAM.[3] Therefore, it could be expected that with functional groups on the amino acid (R_1) and adenosyl portion (R_2) tightly bound to the enzyme surface, the sulfonium center would not be capable of free rotation. In that case it is not unreasonable to find that only one of the two possible isomers at the sulfonium center serves as a methyl donor. The approach of the enzymatically bound nucleophile (denoted by arrows in Chart I) would be predetermined, so that only if the nucleophile and methyl group are properly aligned would methyl transfer occur. Since (+)-L-SAM is enzymatically bound, it could be concluded that there exists sufficient space at this binding site to accommodate the "misplaced" methyl group, but not sufficient flexibility in the enzyme–ligand complex to permit rotation of the sulfonium center into a configuration favorable for methyl transfer. In order to achieve a favorable configuration for methyl transfer in (+)-L-SAM, binding through functional groups in the amino acid (R_1) or adenosyl group (R_2) would have to be sacrificed.

The activity of (+)-L-SAM as an inhibitor of these enzymes, yet its lack of activity as a methyl donor, is of substantial interest, since it points out the fact that by a simple inversion of the configuration at the sulfonium center of L-SAM, the potential for methyl donation is completely lost, while at the same time little is sacrificed in the way of enzymatic binding. These observations demonstrate the strict geometric requirements for the methyl-transfer reaction but, in addition, suggest that there exists some vacant space at the sulfonium binding site to accommodate a "misoriented" methyl group.

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References and Notes

(1) Established Investigator of the American Heart Association.
(2) (a) Abbreviations used are (−)-L-SAM, (−)-*S*-adenosyl-L-methionine; (−)-SAM-$^{14}CH_3$, (−)-*S*-adenosyl-L-methionine-*methyl*-^{14}C; (−)-SAM-$^{14}CO_2H$, (−)-*S*-adenosyl-L-methionine-*carboxyl*-^{14}C; (±)-L-SAM, (±)-*S*-adenosyl-L-methionine; (±)-SAM-$^{14}CH_3$, (±)-*S*-adenosyl-L-methionine-*methyl*-^{14}C; (+)-L-SAM, (+)-*S*-adenosyl-L-methionine; L-SAH, *S*-adenosyl-L-homocysteine; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); PNMT, phenylethanolamine *N*-methyltransferase (E.C. 2.1.1.28); HIMT, histamine *N*-methyltransferase (E.C. 2.1.1.8); HIOMT, hydroxyindole *O*-methyltransferase (E.C. 2.1.1.4); K_i, inhibition constant for the slope. (b) S. K. Shapiro and F. Schlenk, Ed., "Transmethylation and Methionine Biosynthesis", University of Chicago Press, Chicago, Ill., 1965. (c) E. Borek, Ed., "The Biochemistry of *S*-Adenosylmethionine", Columbia University Press, New York, N.Y., in press.
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SAM, at least within physiologic ranges of homocysteine.

Cellular methyltransferases that have been shown experimentally to be inhibited by SAH include catecholamine-*O*-methyltransferase (39), phosphatidylethanolamine methyltransferase (46), histone methyltransferase (18), DNA methyltransferase (18, 26, 47), tRNA and mRNA methyltransferases (48, 49), acetylserotonin methyltransferase (50), and histamine N-methyltransferase (51). The functional consequences of decreased cellular methylation are significant and include central nervous system demyelination (52, 53), reduced neurotransmitter synthesis (39, 50), decreased chemotaxis and macrophage phagocytosis (54, 55), altered membrane phospholipid composition and membrane fluidity (56, 58), altered gene expression (23, 59, 60), and cell differentiation (61, 62). It is likely that the K_i for SAH varies with different cellular methyltransferases and also varies according to tissue priorities and subcellular methyltransferase distribution (63). Tissue levels of SAH reflect the balance between rate of synthesis and the direction of the reversible SAH hydrolase reaction (18). Intracellular SAH can be exported across the plasma membrane against a concentration gradient and appears to be carrier-mediated and largely unidirectional in lymphocytes (63). An increase in SAH has a positive regulatory influence on cystathionine β synthase (64) and methylenetetrahydrofolate reductase activities (65), and SAH has been shown to down-regulate rat liver betaine homocysteine methyltransferase and porcine kidney methionine synthase (66). Taken together, experimental evidence supports a regulatory role for SAH in maintaining normal one-carbon metabolism.

Tissue-specific gene expression depends on the stable inheritance of DNA methylation patterns established during embryogenesis. In differentiated cells, genes are silenced by promoter region methylation in a tissue-specific manner. Disruption of the nonrandom DNA methylation patterns can lead to inappropriate gene expression and promotion of chronic disease (27, 28, 52). Although most cells express genes required for the methionine remethylation, not all cells express genes for the transsulfuration pathway. For example, spleen, adrenal, lung, testes, and heart tissue exhibit negligible cystathionine β synthase activity (67). Thus, tissues lacking appreciable transsulfuration activity might be expected to be most sensitive to increases in SAH and effects on cellular methylation. Of related interest, inactivating mutations in the adenosine deaminase gene lead to severe combined immune deficiency and profound lymphocytopenia. Although the adenosine deaminase enzyme is ubiquitous in cells, the unique sensitivity of lymphocytes may be partly explained by the lack of transsulfuration pathway and increased sensitivity to SAH. Consistent with this notion, resting lymphocytes have been shown to turnover SAM at a rate 3–5 times higher than that estimated for most nonhepatic tissues (68). Further, lymphocyte DNA hypomethylation was recently documented in women undergoing controlled folate depletion (69, 70). Taken together, these observations suggest that global hypomethylation in lymphocyte DNA may be an early biomarker of abnormal methylation in other tissues. Further, the correlation between plasma homocysteine and DNA hypomethylation suggests an indirect mechanism for homocysteine-related disease pathology.

In the present report, the increase in plasma total homocysteine was highly correlated with a parallel increase in SAH; however, no apparent association with SAM was observed. The increase in plasma SAH was also associated with a progressive increase in lymphocyte DNA hypomethylation. It is important to emphasize, however, that the relationship between tissue levels of SAM and SAH and plasma levels of these metabolites is complex and that the tissue-specific origins of plasma SAM and SAH are not known. Interestingly, a modest but significant decrease in plasma methionine levels was associated with the increase in homocysteine. The ratio of the homocysteine to methionine may provide a sensitive clinical biomarker for agents or conditions that compromise methionine synthase activity. For example, a decrease in methionine is consistent with the reduction in methionine synthase activity because of reduced availability of 5-methyltetrahydrofolate. Nutritional folate deficiency has been associated with a decrease in methionine levels (72)[2] and would be expected to decrease the methionine/homocysteine ratio. This ratio may also be useful in the differential diagnosis of genetic aberrations in cytathionine β synthase and MTHFR genes. Both conditions are associated with elevations in tHcy but have opposite effects on methionine. Thus, the ratio would be expected to increase with cytathionine β synthase deficiency and to decrease with MTHFR deficiency.

The lack of correlation between SAM and DNA hypomethylation would suggest that SAM is not a limiting factor for the DNA methyltransferase, at least within physiologic ranges. However, low levels of SAM are clearly associated with up-regulation of the MTHFR enzyme to divert 5,10-methylenetetrahydrofolate toward methionine synthase and its own resynthesis (73). Therefore, rather than an effect on DNA methylation, low SAM levels may have a greater regulatory impact on DNA synthesis by diverting 5,10-methylenetetrahydrofolate away from *de novo* thymidine and purine synthesis. The ability to measure plasma levels of SAM and SAH sensitively and reproducibly should provide new insights into the disregulation of one carbon metabolism in humans.

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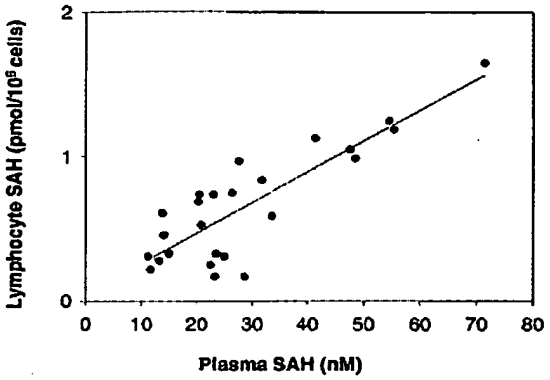

FIG. 3. **Plot of individual values for plasma and intracellular lymphocyte SAH ($r = 0.81$; $p < 0.001$).**

indicated a significant positive correlation between plasma and intracellular SAH concentrations ($r = 0.81$; $p < 0.001$).

Relationship between Mean Plasma SAM and SAH Concentrations and Lymphocyte DNA Methylation—The plasma SAH concentrations and relative levels of DNA methylation were compared between the women with normal levels of plasma tHcy and the women with elevated plasma tHcy. The level of DNA hypomethylation is defined as the extent of [^{3}H]dCTP incorporation into DNA after treatment with the methyl-sensitive restriction enzyme, *Hpa*II, that cuts DNA leaving a guanine overhang at unmethylated recognition sites (33). An increase in radiolabel incorporation reflects the increased number of unmethylated cystosines in DNA. In Fig. 4, a plot of the individual values of plasma SAM and SAH, respectively, are correlated with the extent of lymphocyte DNA hypomethylation. Regression analysis indicated a significant positive association between SAH and DNA hypomethylation ($r = 0.74$, $p < 0.001$); however, there was no apparent correlation between DNA hypomethylation and SAM values. In Fig. 5, the mean SAH values are shown to be increased 2-fold, and DNA hypomethylation increasod 2.6-fold in women with elevated tHcy (range, 9.3–16.6 μM) relative to women with normal tHcy (range, 5.8–8.7 μM).

DISCUSSION

In recent years, a decrease in the ratio of SAM/SAH has been used frequently as a predictor of reduced cellular methylation. In these studies, the decrease in SAM has been emphasized as a limiting cofactor for methyltransferase activity and the major effector of the reduced ratio (36–38). However, earlier studies of alterations in SAM/SAH using nitrous oxide, SAHH inhibition, or cell lines from genetically deficient fibroblasts clearly demonstrated that an increase in SAH, with or without a decrease in SAM, was the more important variable in predicting methyltransferase inhibition and a decrease in cellular methylation (24–26, 39, 40). For example, a decrease in SAM/SAH ratio in the presence of an *increase* or no change in SAM, but significant increase in SAH, was reproducibly associated with hypomethylation and decreased methyltransferase activity (22, 23, 26, 42). It is possible to induce an independent decrease in SAM without a concomitant increase in SAH by genetic or chemical inhibition of methionine adenosyltransferase. Under these conditions, SAM becomes severely depleted below the K_m of most methyltransferases and has resulted in DNA hypomethylation (43) and central nervous system demyelination (44). It is questionable, however, whether physiologic decreases in SAM, such as those induced by nutritional deficiencies, are causally related to cellular hypomethylation. It is

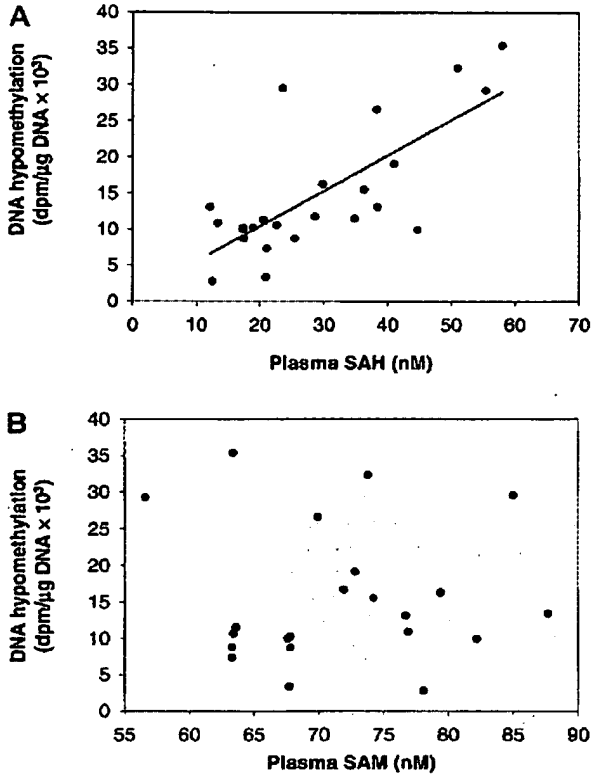

FIG. 4. *A*, plot of individual values for plasma SAH and lymphocyte DNA hypomethylation ($r = 0.74$; $p < 0.001$). *B*, plot of individual values for plasma SAM and lymphocyte DNA hypomethylation.

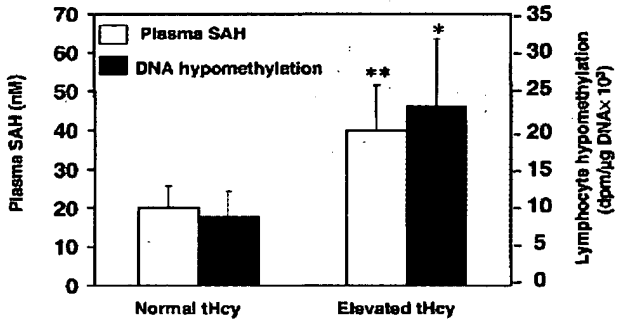

FIG. 5. **Comparison of the mean values of plasma SAH and DNA hypomethylation (means ± S.D.) in women with plasma tHcy concentrations between 5.8 and 8.7 μM and in those with tHcy concentrations between 9.3 and 16.5 μM.** *, $p < 0.05$; **, $p < 0.01$.

important to recognize that the use of the SAM/SAH ratio as a predictor of altered cellular methylation can, in fact, be quite misleading and that evaluation of alterations in individual components may be more informative. For example, it has been shown that identical decreases in SAM/SAH ratio are conditionally associated with reduced methylation capacity depending on the absolute value of SAH (45).[2] Consistent with this concept, the results presented here suggest that an increase in SAH, secondary to an increase in homocysteine, is more strongly correlated with DNA hypomethylation than are alterations in

[2] S. J. James, personal observations.

TABLE I
Plasma levels of homocysteine, methionine, SAH, and SAM in individuals with normal (5.8–8.7 μM) and elevated (9.3–16.5 μM) levels of plasma homocysteine

	Normal tHcy	Elevated tHcy
	mean ± S.D., n = 28	*mean ± S.D., n = 30*
Homocysteine (μM)	7.26 ± 1.11	12.30 ± 1.82*
Methionine (μM)	38.30 ± 9.71	26.80 ± 6.25*
Homocysteine/methionine ratio	0.20 ± 0.05	0.50 ± 0.17*
SAM (nM)	79.90 ± 8.81	76.41 ± 6.13
SAH (nM)	20.00 ± 5.55	40.10 ± 12.5*
SAM/SAH ratio	4.43 ± 1.48	2.40 ± 1.28*

* $p < 0.001$ as compared to group with normal homocysteine.

and a pressure of 100–110 kgf/cm^2 (1500–1800 psi). tHcy, methionine, SAM, and SAH were quantified using a model 5200A Coulochem II electrochemical detector (ESA, Inc.) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). Methodologic details have been described previously (32).

Lymphocyte Global DNA Methylation Using Cytosine Extension Assay—Assessment of lymphocyte DNA methylation was accomplished using the cytosine extension assay previously described in detail (33). Briefly, ~1 μg of genomic DNA was digested for 16–18 h with 20 units of *Hpa*II according to manufacturer's protocol (New England Biolabs, Beverly, MA). A second DNA aliquot served as background control and was similarly incubated without addition of restriction enzyme. The single nucleotide extension reaction was performed in a 25-μl reaction mixture containing 0.5 μg of DNA, 1× polymerase chain reaction buffer II, 1.0 mM $MgCl_2$, 0.25 units of AmpliTaq DNA polymerase (Perkin-Elmer), and 0.1 μl of [^{3}H]dCTP (57.4 Ci/mmol, NEN Life Science Products), incubated at 56 °C for 1 h, and then placed on ice. Duplicate 10-μl aliquots from each reaction were applied onto Whatman DE-81 ion exchange filters and washed three times with 0.5 M sodium phosphate buffer (pH 7.0) at room temperature. Filters were dried and processed for scintillation counting in 10 ml of Ultima Gold (Packard Bioscience Co., Meriden, CT). Background radiolabel incorporation in untreated samples is subtracted from enzyme-treated samples, and the results are expressed as relative [^{3}H]dCTP incorporation/0.5 μg DNA.

Statistics—Data are presented as the means ± S.D. Statistical differences between means were calculated using the Student's *t* test and Sigmastat software (Jandel Scientific, San Rafael, CA).

RESULTS

Mean Plasma Concentrations of Methionine, SAM, and SAH in Women with Normal and Elevated tHcy Concentrations—In Table I, mean values for plasma homocysteine, methionine, SAM, and SAH are shown as a function of fasting plasma tHcy levels in the 58 participants. The women were stratified by tHcy based on previously published normal ranges for adult females (34, 35). In this cohort, women with tHcy ranging from 5.8 to 8.7 μM (mean, 7.26 ± 1.1) were designated to be within the "normal" range of tHcy and women with tHcy ranging from 9.3 to 16.5 μM (mean, 12.3 ± 1.82) were designated as having "elevated" tHcy concentrations. Among the women within the normal range of tHcy, the mean plasma SAM concentration was 79.9 ± 8.81 nM, the mean SAH concentration was 20.0 ± 5.55 nM, and the SAM/SAH ratio was 4.43 ± 1.48. Among the women with elevated tHcy, plasma methionine concentrations were significantly decreased, and the ratio of tHcy/methionine was significantly increased relative to the women within the normal tHcy range ($p < 0.001$). Elevated plasma tHcy was not associated with an alteration in SAM levels, but SAH levels were increased 2-fold relative to women with normal tHcy, and the SAM/SAH ratio was decreased by one-half ($p < 0.001$).

Relationship between Plasma Homocysteine and Plasma Levels of SAM, SAH, and Methionine—Fig. 2*A* is a plot of the individual values of plasma tHcy and the corresponding plasma methionine values. Fig. 2*B* is a similar plot showing the relationship between plasma tHcy and SAH for each participant, and Fig. 2*C* shows the relationship between plasma tHcy and SAM. A modest but significant negative correlation was found between plasma tHcy and methionine ($r = 0.50$; $p < 0.01$). Increasing concentrations of plasma tHcy were strongly associated with increased concentrations of plasma SAH ($r = 0.73$; $p < 0.001$), whereas there was no apparent relationship between plasma SAM and tHcy. A strong negative correlation was found between tHcy and the ratio of SAM/SAH ($r = 0.73$, $p < 0.01$; data not shown); the decrease in SAM/SAH ratio was due to the increase in SAH in all cases.

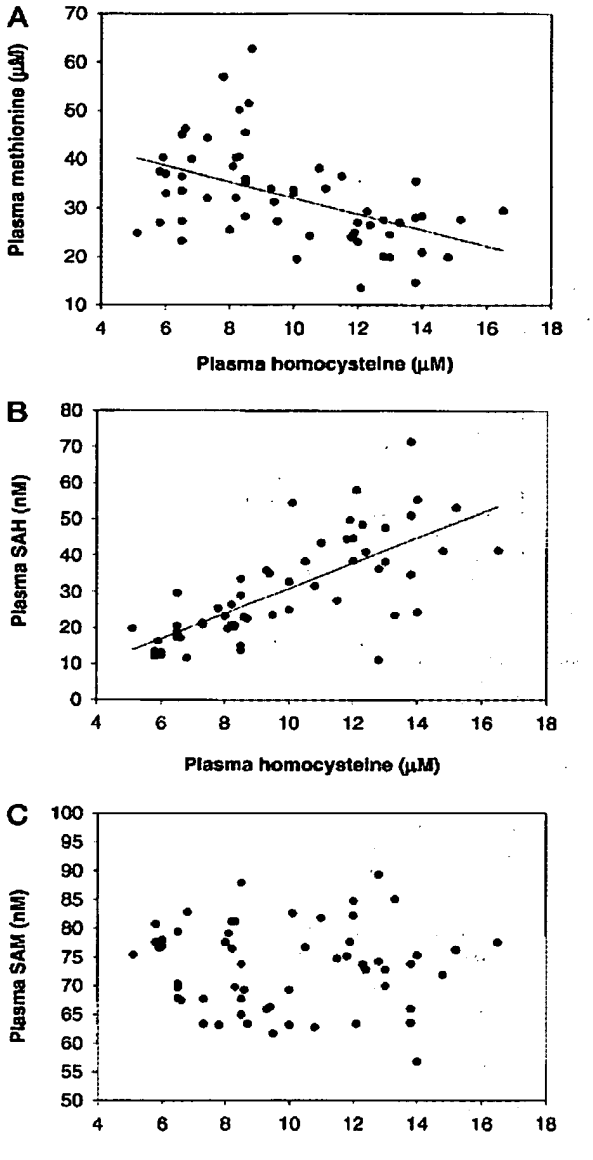

FIG. 2. *A*, plot of individual values for plasma tHcy and methionine for each participant ($r = 0.50$; $p < 0.01$). *B*, plot of individual values for tHcy and SAH ($r = 0.73$; $p < 0.001$). *C*, plot of individual values of tHcy and SAM.

Relationship between Plasma SAH and Intracellular Lymphocyte SAH—Intracellular SAH concentration was determined in extracts of fresh lymphocytes isolated from a subset of the participants. Fig. 3 is a plot of individual plasma SAH concentration and the corresponding intracellular lymphocyte SAH concentration for each individual. Regression analysis

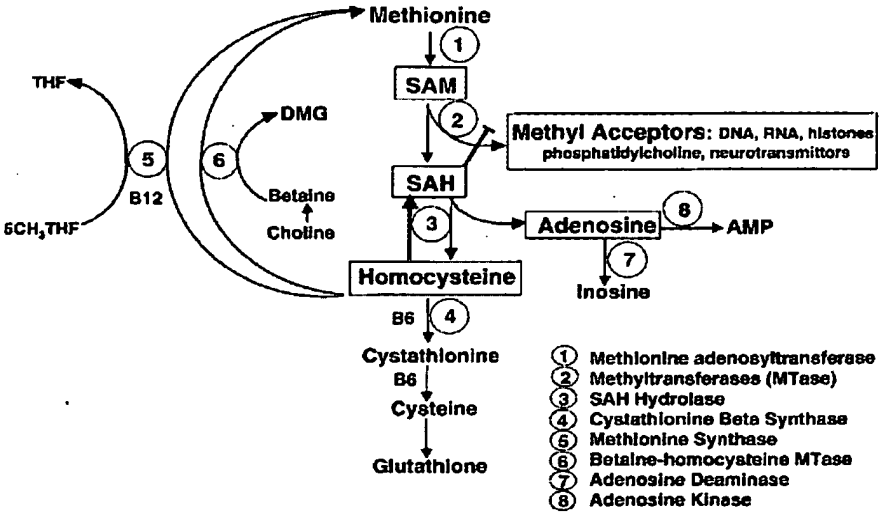

FIG. 1. **An overview of one-carbon metabolism with emphasis on the reversible SAH hydrolase reaction (3).** The hydrolysis of SAH is dependent on product removal of homocysteine and adenosine. In the absence of efficient product removal, SAH accumulation can inhibit methyltransferase activity by high affinity binding to the enzyme active site.

Under normal physiologic conditions, SAH is hydrolyzed by SAHH to adenosine and homocysteine. It is important to note, however, that this reaction is readily reversible with equilibrium dynamics that strongly favor SAH synthesis rather than hydrolysis. In fact, the only reason that this reaction proceeds in the hydrolytic direction is efficient product removal (21). Thus, metabolic perturbations that interfere with the efficient removal of homocysteine and adenosine will lead to an increase in SAH (22). The existence of multiple routes of removal for both these metabolites is consistent with the necessity for efficient product removal to avoid SAH accumulation and the potentially negative consequences of methyltransferase inhibition (1, 23). Homocysteine can be methylated to regenerate methionine in all cells by the folate/B_{12}-dependent methionine synthase reaction and additionally by the betaine-homocysteine methyltransferase reaction in liver and kidney of humans (1). A third route of homocysteine removal is the irreversible pyridoxal phosphate-dependent transsulfuration pathway in which cystathionine β synthase and lyase reactions permanently remove homocysteine from the methionine cycle. Adenosine can be efficiently removed by either the adenosine deaminase reaction or the adenosine kinase reaction. Experimental studies have shown that analog inhibition of these pathways or genetic deficiencies in these enzymes results in SAH accumulation and potent inhibition of methyltransferases (24–26).

In the present report, using a sensitive new method for measuring plasma concentrations of SAM and SAH, we show for the first time that modorate elevation in plasma total homocysteine concentration is positively associated with parallel increases in plasma SAH concentrations and lymphocyte DNA hypomethylation. These data support an indirect mechanism for homocysteine pathogenicity secondary to SAH-mediated inhibition of the DNA methyltransferase. The disruption of the heritable methylation patterns in DNA can lead to alterations in chromatin structure and alterations in gene expression that can promote chronic disease states (27–30).

MATERIALS AND METHODS

Reagents—SAM, SAH, trichloroacetic acid, sodium phosphate monobasic, monohydrate, and 1-heptanesulfonic acid were obtained from Sigma. HPLC grade methanol was purchased from J. T. Baker Inc. (Phillisburg, NJ). Deionized HPLC-grade water for HPLC was prepared by passage through a Syrbon/Barusted NANOpure II filtration system (Boston, MA) and subsequent passage through a C_{18} Sep-Pak cartridges (Millipore Corp., Milford, MA).

Subjects and Blood Collection—Participants were 58 healthy adult females with a mean age of 37.2 years (range, 19–53 years) who had participated in a previous clinical study (15). Fasting blood samples were collected into EDTA-Vacutainer tubes, immediately chilled on ice, and centrifuged at 400 × *g* for 15 min at 4 °C. Aliquots of the plasma layer were transferred into multiple cryostat tubes and stored at −20 °C until analysis. Individual aliquots were thawed for determination of plasma homocysteine, methionine, SAM, and SAH. DNA was extracted from the cell pellet using standard chloroform/phenol methodology (31). In a subset of women, mononuclear cells were immediately isolated by carefully layering whole blood onto an equal volume of Histopaque® 1077 (Sigma) at room temperature and centrifuging at 400 × *g* for 30 min. Mononuclear cells were recovered from the interface and washed several times as described by the manufacturer, and aliquots of approximately 10^9 cells were homogenized in 200 μl of phosphate-buffered saline. The homogenate was centrifuged at 18,000 × *g* for 1 min, and the supernatant was stored at −80 °C until HPLC analysis.

Sample Preparation—For determination of total homocysteine (tHcy) and methionine, 50 μl of freshly prepared 1.43 M sodium borohydride solution containing 1.5 μM EDTA, 66 mM NaOH, and 10 μl of *n*-amyl alcohol were added to 200 μl of plasma or cell homogenate. After gentle mixing, the solution was incubated in 40 °C water bath for 30 min with gentle shaking. To precipitate proteins, 250 μl of ice-cold 10% *meta*-phosphoric acid was added, and the sample was incubated for 10 min on ice. After centrifugation at 18,000 × *g* for 15 min at 4 °C, the supernatant was filtered through a 0.2-μm filter (PGC Scientific, Frederick, MD), and a 20-μl aliquot was injected into the HPLC system. For determination of SAM and SAH, 40 μl of 40% trichloroacetic acid were added to 200 μl of plasma or cell extract to precipitate protein, mixed well, and incubated on ice for 30 min. After centrifugation for 15 min at 18,000 × *g* at 4 °C, supernatants containing SAM and SAH were passed through a 0.2-μm filter, and 20 μl was injected into the HPLC system.

HPLC Chromatography—The elution of homocysteine and methionine utilized a different mobile phase than that used for elution of SAM and SAH; however, both analyses were accomplished using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C_{18} column (5 μm; 4.6 × 150 mm, MCM, Inc., Tokyo, Japan) obtained from ESA, Inc. (Chelmsford, MA). A 20-μl aliquot of plasma or cell extract was directly injected onto the column using a Beckman autosampler (model 507E). To assure standardization between sample runs, calibration standards and reference plasma samples were interspersed at intervals during each run. For elution of homocysteine and methionine, the mobile phase consisted of 50 mM sodium phosphate monobasic monohydrate, 1.0 mM ion-pairing reagent octane sulfonic acid, 2% acetonitrile (v/v) adjusted to pH 2.7 with 85% phosphoric acid, with isocratic elution at ambient temperature at a flow rate of 1.0 ml/min and a pressure of 120–140 kgf/cm² (1800–2100 psi). For elution of SAM and SAH, the mobile phase consisted of 50 mM sodium phosphate monobasic, monohydrate, 10 mM 1-heptanesulfonic acid, 7.5% (v/v) methanol adjusted to pH 3.4 with 85% phosphoric acid, with isocratic elution at ambient temperature at a flow rate of 1.0 ml/min